

The cell biology and genome of *Stentor pyriiformis*, a giant cell that embeds symbiotic algae in a microtubule meshwork

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ABSTRACT Endosymbiotic events in which an endosymbiont is retained within a cell that remains capable of phagocytosis, a situation known as mixotrophy, provide potentially important clues about the eukaryotic evolution. Here we describe the cell biology and genome of the giant mixotrophic ciliate *Stentor pyriiformis*. We show that *S. pyriiformis* contains *Chlorella variabilis* as an endosymbiont that retains the ability to live outside the host. Within the host, the *Chlorella* cells surrounded by microtubule “baskets” near the cell surface. Photosynthetic efficiency of the *Chlorella* is reduced inside the *Stentor* cell compared with outside the host, due to increased nonphotochemical quenching. *S. pyriiformis* displays positive phototaxis via directed swimming that requires the presence of the *Chlorella*, implying a potential flow of information from the symbiont to direct the orientation and swimming of the host cell. We sequenced the *S. pyriiformis* genome and found that it employs a standard genetic code, similar to other *Stentor* species but different from most other ciliates. We propose that *S. pyriiformis* will serve as a useful model system for studying endosymbiosis, with unique advantages in terms of size and regenerative ability as well as distinct cellular and genomic features compared with other mixotrophic ciliate models.

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SIGNIFICANCE STATEMENT

- Mixotrophic cells allow cellular adaptations necessary for endosymbiosis in the “phagocytosis first” model to be investigated in extant organisms.
- *Stentor pyriiformis*, a mixotrophic ciliate that is highly diverged from the standard model *Paramecium bursaria*, was found to anchor the symbionts in a meshwork of microtubules, perform phototaxis by directed swimming, and display reduced photosynthetic efficiency of algae when inside the host. The genome of *S. pyriiformis* uses a standard genetic code.
- *S. pyriiformis* provides a new window into how cells may adapt to contain other cells inside of themselves.

INTRODUCTION

The evolution of the eukaryotic cell is a history of one unicellular organism incorporating parts of another to produce a new organism with improved capabilities. Mitochondria and chloroplasts evolved from free-living organisms that became internalized and converted into organelles dependent on the host cell (Sagan, 1967; López-García and Moreira, 2015; Martin et al., 2015; Vosseberg et al., 2024). The first eukaryotic common ancestor (FECA) somehow engulfed another organism without digesting it, after which many more steps of gene transfer to the host cell fixed the internalized cell as an organelle (Maciszewski and Karnkowska, 2019). These gene transfer processes, with their accompanying changes in protein trafficking and targeting, potentially combined with further uptake events, ultimately gave rise to the last eukaryotic common ancestor (LECA) from which all current eukaryotic cells evolved (Richards et al., 2024). There are various scenarios for how the first eukaryotic cells might have formed. One scenario (known as “phagocytosis first”) suggests that the free-living precursor cells that eventually gave rise to mitochondria were ingested by a cell that already contained the necessary phagocytic machinery (Yutin et al., 2009; Guy and Ettema, 2011), but instead of being digested, they were maintained as endosymbionts which then gradually became more dependent on the host cell until they could no longer exist as free-living organisms and thus became fixed as organelles. This scenario has been questioned because phagocytosis would be difficult as long as the electron transport chain is embedded in the cell membrane (Martin et al., 2017). Two alternative scenarios have been proposed: in one potentially far-fetched case, one cell type ends up inside another due to some catastrophic event, after which the two cell types need to adapt to the new arrangement. In a second alternative scenario, known as “pre-symbiosis” or “symbiosis first” (Speijer, 2020), two cell types develop a symbiotic relationship in which they grow together in close proximity (Dey et al., 2016), and then when one of the cell types evolves the ability to engulf the other, the original symbiotic relationship becomes one of endosymbiosis.

In evaluating these scenarios, it is of great interest to know more about how endosymbiosis can occur, and what cellular or genomic adaptations it may require in the host and symbiont. It is impossible to go back in time to observe what took place during the evolution of FECA and LECA. However, one way to gain insights into

potential sequences of events and accompanying mechanisms is to examine extant species in which one cell maintains another cell type within it as an endosymbiont, but where the endosymbiont has retained its ability to live independently of the host and has thus not yet become fixed as an organelle.

Given the importance of determining the relation between phagocytic uptake, endosymbiosis, and eventual organelle fixation, a particularly interesting phenomenon that can still be observed in extant organisms is mixotrophy, in which a phagocytic host cell acquires endosymbionts and maintains them inside itself, while still retaining the ability to capture prey via phagocytosis (Esteban et al., 2010). Such mixotrophic organisms can take up new symbionts by phagocytosis without digesting them, instead maintaining them as endosymbionts. In many cases, the ingested organism is retained fully intact inside perialgal vesicles, while in other cases the plastids are extracted and retained by the mixotrophic host (Johnson, 2011). Successful endosymbiosis of intact algal cells inside a phagocytic host requires that the endosymbiont escape digestion and coordinate its division and metabolism with that of the host cell. Meanwhile, the host cell is still capturing prey by phagocytosis, thereby supplementing not only its own nutritional requirements, but also those of the endosymbionts.

The cell biology of mixotrophy raises many interesting questions about how one cell interacts stably with another, for example: what structural adaptations might the host evolve to better maintain the endosymbiont; does the endosymbiont alter its metabolic functions in a way that would benefit the host; and, to what extent does information flow from the symbiont to the host to direct its behavior? Understanding how the host and symbiont cells change their structure and function to work together may provide clues about the structural and regulatory plasticity of cells in general, and may also offer a glimpse into what may have been an important intermediate stage of organelle fixation.

Mixotrophic endosymbiosis with algal cells is seen in many ciliates (Dolan, 1992; Esteban et al., 2010), the most classical example being *Paramecium bursaria* (Kodama and Fujishima, 2010; He et al., 2019; Jenkins, 2024), which contains algal cells of the species *Chlorella variabilis* as endosymbionts. As with other ciliates that contain algal cells as endosymbionts, the *Chlorella* cells inside *P. bursaria* retain the ability to grow on their own outside the host, and can be reintroduced to host cells from which algae have been removed, the latter also being able to live freely without their symbionts (Kodama and Fujishima, 2010). *Chlorella* enter the host cell via phagocytosis, and end up in perialgal vesicles, which are derived from digestive vacuoles that first surround the algae when they are taken up from the environment (Fujishima and Kodama, 2022; Kodama and Sumita, 2022). While inside the host, the cell walls of *Chlorella* are thinner than when the *Chlorella* are free-living (Higuchi et al., 2018), possibly reflecting the activity of glycan-processing enzymes encoded by the *P. bursaria* genome (Jenkins et al., 2024). The perialgal vesicles are associated with the cell cortex, showing close association with mitochondria and trichocysts (Kodama and Fujishima, 2023).

Chlorella cells can divide while inside the *Paramecium* host, and their division is correlated with the division of the host cell (Kadono et al., 2004; Takahashi et al., 2007). The period when the algal cells proliferate matches a period in *Paramecium* division when cytoplasmic streaming pauses. This streaming is microtubule dependent (Nishihara et al., 1999), and inhibition of streaming using microtubule inhibitors is sufficient to trigger *Chlorella* proliferation (Takahashi et al., 2007), pointing to a role for host microtubules in regulating algal replication.

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Abbreviations used: APS, ammonium persulfate; BSA, bovine serum albumin; BUSCO, benchmarking universal single-copy orthologs; CTAB, cetyltrimethylammonium bromide; CVDE, chlorophyllase violaxanthin de-epoxidase; DCMU, N-(3,4-dichlorophenyl)-N-dimethylurea; EtOH, ethyl alcohol; FECA, first eukaryotic common ancestor; FPSII, photosystem II efficiency kb kilobase; LECA, last eukaryotic common ancestor; LED, light emitting diode; LHCSR, stress-related light-harvesting complex Mb megabase; MBBM, modified bold's basal medium; MCT, microcentrifuge tubes; MSM, modified stentor medium n.a. numerical aperture; NaCl, sodium chloride NPQ non photochemical quenching nt nucleotide; PAM, pulse amplitude modulated; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PSII, photosystem two; qE, energy dependent quenching; RT, room temperature; SDS, sodium dodecyl sulfate; TAP, Tris acetate phosphate; TEMED, Tetramethylethylenediamine; tRNA, transfer ribonucleic acid; ZEP, zeaxanthin epoxidase.

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P. bursaria is just one of a large number of mixotrophic ciliates. Many retain their endosymbionts in intact form, some retain just the plastids, and in the case of the marine ciliate *Mesodinium*, the retained plastids can undergo division inside the hosts supported by genetic information from nuclei that were ingested and then maintained along with the chloroplasts from the prey (Moeller and Johnson, 2023). The ingestion and maintenance of one organism inside another may seem unusual, but in some fresh water aquatic environments, mixotrophic ciliates constitute the majority of ciliate biomass (Finlay et al., 1988; Woelfl and Geller, 2002), suggesting that mixotrophy has proven to be a highly successful evolutionary strategy.

The fact that many ciliates retain their algal endosymbionts in an intact form, capable of living outside the host, rather than becoming fixed as organelles, suggests that the endosymbionts are not transferring critical genes to the host nucleus. Although ciliates are in some cases capable of horizontal gene transfer (Ricard et al. 2006; Archibald, 2008; Shaw et al., 2010; Feng et al., 2020), in species like *P. bursaria* this does not seem to have taken place from the *Chlorella* genome, as judged by the fact that the endosymbionts are still capable of living outside the host and therefore have not lost critical genes (Kodama and Fujishima, 2010). Why have not these algae become fixed as organelles?

One potential hypothesis is that because most ciliates use a nonstandard genetic code in which one or more stop codons specify amino acids (reviewed in Koonin and Novozhilov, 2017), for example UGA encoding tryptophan, gene transfer from an endosymbiont using a standard code to a host nucleus using an alternative code might be more difficult to achieve in the ciliates compared with other phyla. The recent assembly of the *P. bursaria* genome confirms that it employs a nonstandard genetic code and small introns, with increased intron retention of large introns suggesting a bias against correct processing for introns of more standard length (Leonard et al., 2024). On the other hand, there is evidence that *P. bursaria* or its ancestor has undergone horizontal gene transfer from prokaryotes (Jenkins et al., 2024) so there does not seem to be an insurmountable obstacle to such transfer.

Currently, *P. bursaria* is by far the most extensively studied mixotrophic ciliate at both the cell biological and genomic levels. The fact that most of what we know about cellular and genomic adaptations to mixotrophy in ciliates comes from a single organism makes it hard to know which of the features outlined above may be general features important for mixotrophic interactions with *Chlorella* and which may be mere historical accidents acquired in the lineage of this one organism. By exploring the cell biology of endosymbiosis in other, more distantly related ciliates, in which the acquisition of *Chlorella* endosymbionts is likely to have occurred independently, it would become possible to ask whether any of the specific cell biological features of host–symbiont interaction seen in *P. bursaria* are employed in other independent cases, which would suggest that endosymbiosis may have relied on a common underlying capability shared among ciliates.

Ciliates are classified into two major groups, Postciliodesmatophora and Intramacronucleata (Lynn, 2010). *P. bursaria* and indeed most of the mixotrophic ciliate model systems that have been studied, such as *Euplotes*, fall into the Intramacronucleata subphylum. Postciliodesmatophora consists of two classes—Karyorelictea and Heterotrichea, the latter being distinguished by ultrastructural features and an unusually large cell size. This group includes the genus *Stentor* (Tartar, 1961), famous for their remarkable abilities to heal wounds and regenerate (Marshall, 2021), as well as *Spirostomum* (Zhang et al., 2023) and *Blepharisma* (Singh et al., 2023).

All of the Intramacronucleata, as well as *Blepharisma*, use a non-standard genetic code. In contrast, the first *Stentor* species whose genome was determined, *Stentor coeruleus*, uses a standard genetic code in which all three stop codons are used as stop codons (Slabodnick et al., 2017).

Here, we describe the cellular organization, behavior, and genome of *Stentor pyriformis*, a giant heterotrichous ciliate that, like *P. bursaria*, also hosts *C. variabilis* endosymbionts (Hoshina et al., 2021). There are several reasons for wanting to investigate endosymbiosis in this organism. First, as argued above, understanding the cellular features that enable mixotrophy in a ciliate more distantly related to *P. bursaria* may allow general and essential features to be distinguished from historical accidents. Second, *S. pyriformis* provides an opportunity to examine mixotrophy in ciliates with a standard genetic code. Finally, *S. pyriformis* offers some specific experimental opportunities due to their large size, robust wound-healing, and regeneration capacity, similar to other *Stentor* species, which opens the possibility of microinjection and microsurgical techniques to manipulate the host cell and its symbionts, as well as their tractability for measurement using spectroscopic assays for photosynthetic efficiency and the ease of determining cell orientation during phototaxis.

Our results show that *S. pyriformis* cells construct a system of microtubule structures to hold the *Chlorella* at the cell cortex; that photosynthetic efficiency of the *Chlorella* is reduced when inside the host, possibly playing a role in light protection; that phototaxis in *S. pyriformis* occurs via directional swimming that requires the presence of photosynthetically active endosymbionts, suggesting an exchange of information between the two; and that the macronuclear genome of *S. pyriformis* uses a standard genetic code and has a substantially smaller genome than *S. coeruleus*, suggesting it might not have undergone the whole-genome duplications seen in other members of the *Stentor* genus. Taken together, our results show that some aspects of the symbiosis in *S. pyriformis*, such as the type of endosymbiont *C. variabilis* and docking of endosymbionts near the cell surface, are highly similar to those in *P. bursaria*, while other aspects, such as the standard genetic code of the host and the use of directional phototaxis versus kinetic accumulation, are notably different, confirming that the use of *S. pyriformis* as a model system can lead to new questions about host–symbiont interactions at the cellular level that may be significant for the initial, and still ongoing, evolution of eukaryotic cells.

RESULTS

Cell anatomy of *S. pyriformis*

We identified cells of *S. pyriformis* in a lake in Falmouth, MA, based initially on the appearance of the cells as large (300 μm diameter) green trumpet-shaped cells (Figure 1A; Supplemental Video S1), sometimes swimming near the surface and sometimes attached to plants in shallow water. Like other *Stentor* species, one end of the cell contains a large membranellar band consisting of thousands of cilia (Figure 1B). The cells were covered in longitudinal bundles of microtubules (Figure 1B) corresponding to the microtubule bundles known as Km fibers in other *Stentor* species, and contained roughly 5000 green algal cells, based on an automated cell counter measurement of algae isolated from five cells. The algal cells have diameters of $\sim 3 \mu\text{m}$ and are mostly located near the cell surface (Figure 1C).

DAPI staining revealed a macronucleus organized as a small number of spherical nodes, often as few as one or two, which is

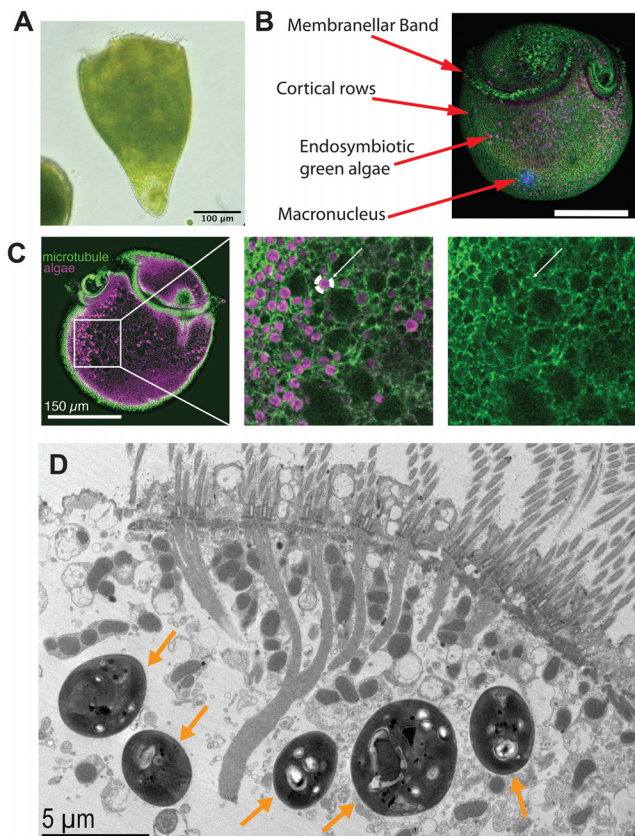


FIGURE 1: Anatomy of *S. pyriformis*. (A) Live *S. pyriformis* cell imaged by transmitted light microscopy. (B) Immunofluorescence image of *S. pyriformis*, (green) tubulin immunofluorescence, (pink) algal chlorophyll autofluorescence. Scale bar, 250 μ m. (C) Section through 3D immunofluorescence image showing detail of algal cells docked near cell cortex surrounded by microtubules. (D) Thin section electron micrograph of *S. pyriformis* showing algal endosymbionts (orange arrows) in association with cortical structures. Host cell cilia are visible in the upper right corner, and the branching structure is the cortical rootlet system of the membranellar band.

distinct from the moniliform macronucleus seen in *S. coeruleus* (Tartar, 1961; McGillivray et al., 2023) but is in line with the earliest reports of *S. pyriformis* structure which reported a macronucleus containing two large, spatially separated nodes (Johnson, 1893) as well as a more recent report on *S. pyriformis* (Hoshina et al., 2021). Tubulin immunofluorescence revealed a dense meshwork of microtubules surrounding the algal endosymbionts at the cell surface, creating an impression of microtubule-based “baskets” surrounding each endosymbiont (Figure 1C).

At the ultrastructural level, thin-section electron microscopy of chemically fixed cells showed that the algae inside perialgal vacuoles were interspersed with mitochondria at the cell surface (Figure 1D). The endosymbionts contained a prominent pyrenoid with thylakoid membranes penetrating it, as previously reported in *S. pyriformis* isolated from highland bogs in Japan (Hoshina et al., 2021).

Host and symbiont phylogeny in *S. pyriformis*

For the vast majority of mixotrophic ciliates that contain green algal endosymbionts, the symbiont is a species of *Chlorella*. However, different freshwater ciliates can contain different species of *Chlorella* endosymbionts (Summerer et al., 2008). A study of *S.*

pyriformis cells isolated in Japan identified the endosymbiont as *C. variabilis* (Hoshina et al., 2021). We sequenced PCR-amplified 18S rDNA and found that the endosymbiont in our cells isolated in Falmouth, MA are also *C. variabilis* (Figure 2A). We confirmed this identification by sequencing a PCR-amplified region of the chloroplast-encoded *rbcL* gene (Figure 2B). The *Chlorella* cells can be readily obtained by lysing *S. pyriformis* cells using a glass rod and then spreading the contents of the cell on a Modified Bold's Basal Medium (MBBM) agar plate, after which the *Chlorella* can be grown, independently of the host, on MBBM media (Figure 2C).

In *P. bursaria*, which also contains *C. variabilis*, the endosymbionts have cell walls that are approximately half as thick as when they are in the free-living state (Higuchi et al., 2018). Our thin-section transmission electron micrographs of chemically fixed *S. pyriformis* cells indicate that the cell wall in the *Chlorella* inside *S. pyriformis* are ~30–40 nm thick (Figure 2, D and E), which is comparable to the thickness of cell walls seen in *Chlorella* inside *P. bursaria* (Kodama and Fujishima, 2010).

We initially identified the organism that we collected as *S. pyriformis* based on its size, shape, and presence of green endosymbionts, all of which match historical and modern descriptions (Johnson, 1893; Hoshina et al., 2021). In parallel with phylogenetic analysis of the *Chlorella* endosymbionts, we carried out 18S analysis of the host, which confirmed that we are in fact working with *S. pyriformis* (Figure 2F).

Microtubule baskets surround algal vesicles at the cell cortex in *S. pyriformis*

Immunofluorescence images show that algal cells at the cell cortex are surrounded by a meshwork of microtubules that extends ~3–4 μ m into the cell, which is comparable to the diameter of the *Chlorella* cells (Figure 3, A and B). Counting *Chlorella* cells in sections from three-dimensional (3D) images indicates that more than 90% of algal cells are associated with microtubule “baskets.” We obtained higher-resolution images of the microtubule baskets using expansion microscopy (Figure 3C; Supplemental Video S2). These images show that the “baskets” do not have any obvious regular structure but appear more like a meshwork of microtubules with gaps where the algae are located.

The interaction of the endosymbionts in their perialgal vacuoles with the microtubule basket system is highly dynamic. In live images, the symbionts can be seen to move over the surface (Supplemental Video S3), and they sometimes disappear from surface patches, suggesting a continuous remodeling of the microtubule network. When cells are centrifuged at 4000 $\times$ g, the algal cells detach from the cortex and pile up on one side of the cell. After such a perturbation, the algae return to the cortex on the time scale of tens of minutes, again consistent with a dynamic interaction with the cortical microtubule network. A similar ability of *Chlorella* endosymbionts to be displaced by centrifugation and then reattach to the cortex has been shown in *P. bursaria* (Kodama and Fujishima, 2013; 2023).

Clearing of *Chlorella* endosymbionts from the *Stentor* host

To obtain *Stentor* host cells that were free of endosymbionts, we developed a protocol in which cells are centrifuged at 10,000 $\times$ g, which causes the alga to accumulate on one side of the cell (Figure 4A). Upon centrifugation for 1 min, cells burst and release their algal contents. Due to the well-known wound-healing ability of *Stentor* cells (Marshall, 2021; Zhang et al., 2021), the host cells are not killed by this procedure, which results in actively swimming alga-free host cells. As an alternative approach, it is also possible to

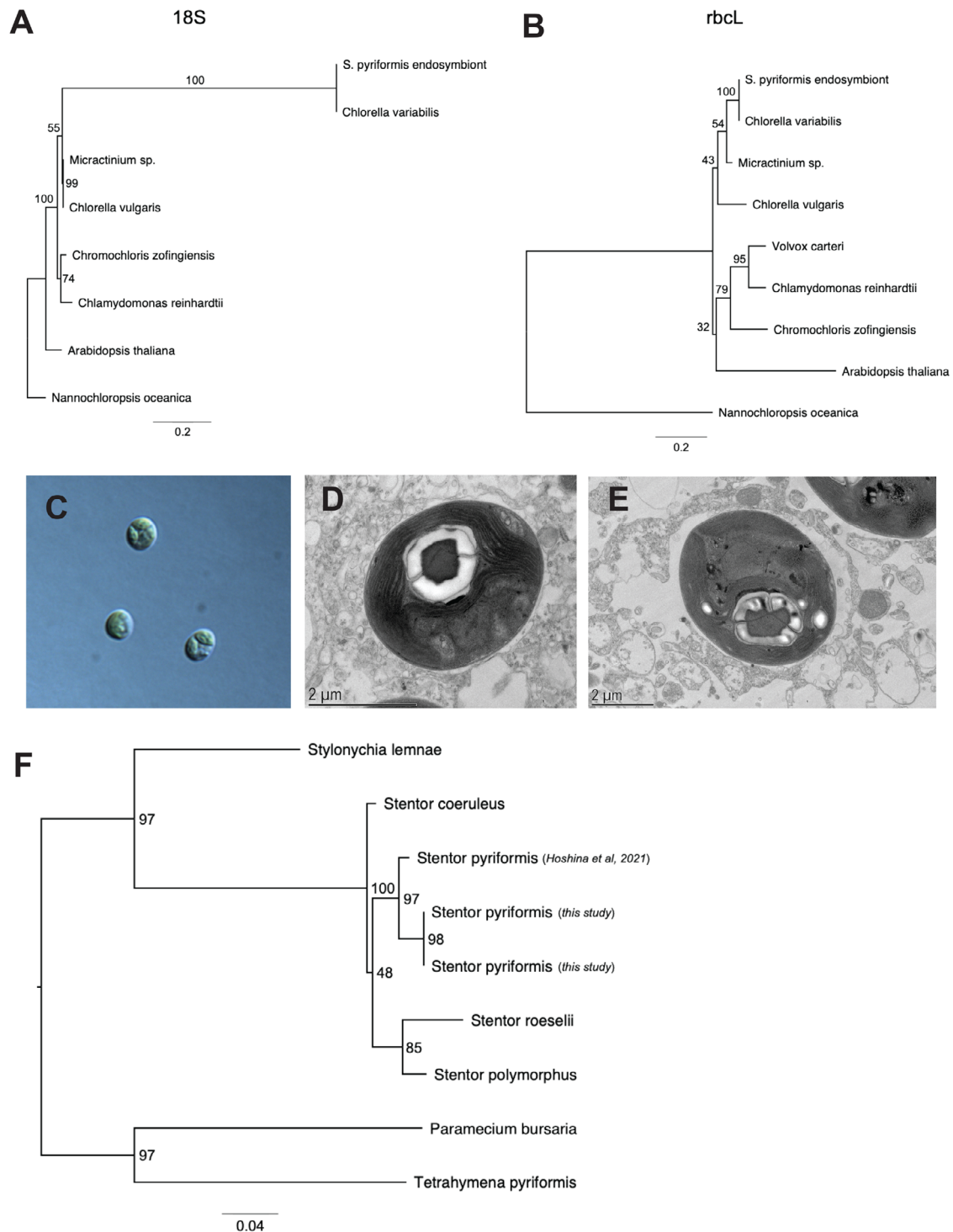


FIGURE 2: Characterization of the endosymbiont in *S. pyriformis*. (A) Analysis of 18S rDNA sequence indicates that endosymbiont is *C. variabilis*. (B) Identity of symbiont confirmed by sequence of the *rbcL* gene. (C) Image of live *Chlorella* cells isolated from *S. pyriformis*. (D, E) Thin section electron micrographs showing detail of *Chlorella* ultrastructure. (F) Analysis of 18S rDNA sequence confirms that the cells isolated from Falmouth, MA, are indeed *S. pyriformis*.

bisect cells with a glass needle after the algae have been collected on one side by centrifugation (Figure 4B). The resulting alga-free cells are white in color, thus apparently lacking the blue or purple pigments found in pigmented *Stentor* species like *S. coeruleus* or *S. amethystinus*. In other *Stentor* species (Yang et al., 1986) as well

as other pigmented ciliates (Finlay and Fenchel, 1986), the pigments are thought to produce reactive oxygen in the presence of light. The absence of pigment in *S. pyriformis* host cells may thus be related to their need to expose themselves to sunlight in order to allow effective photosynthesis by the endosymbionts.

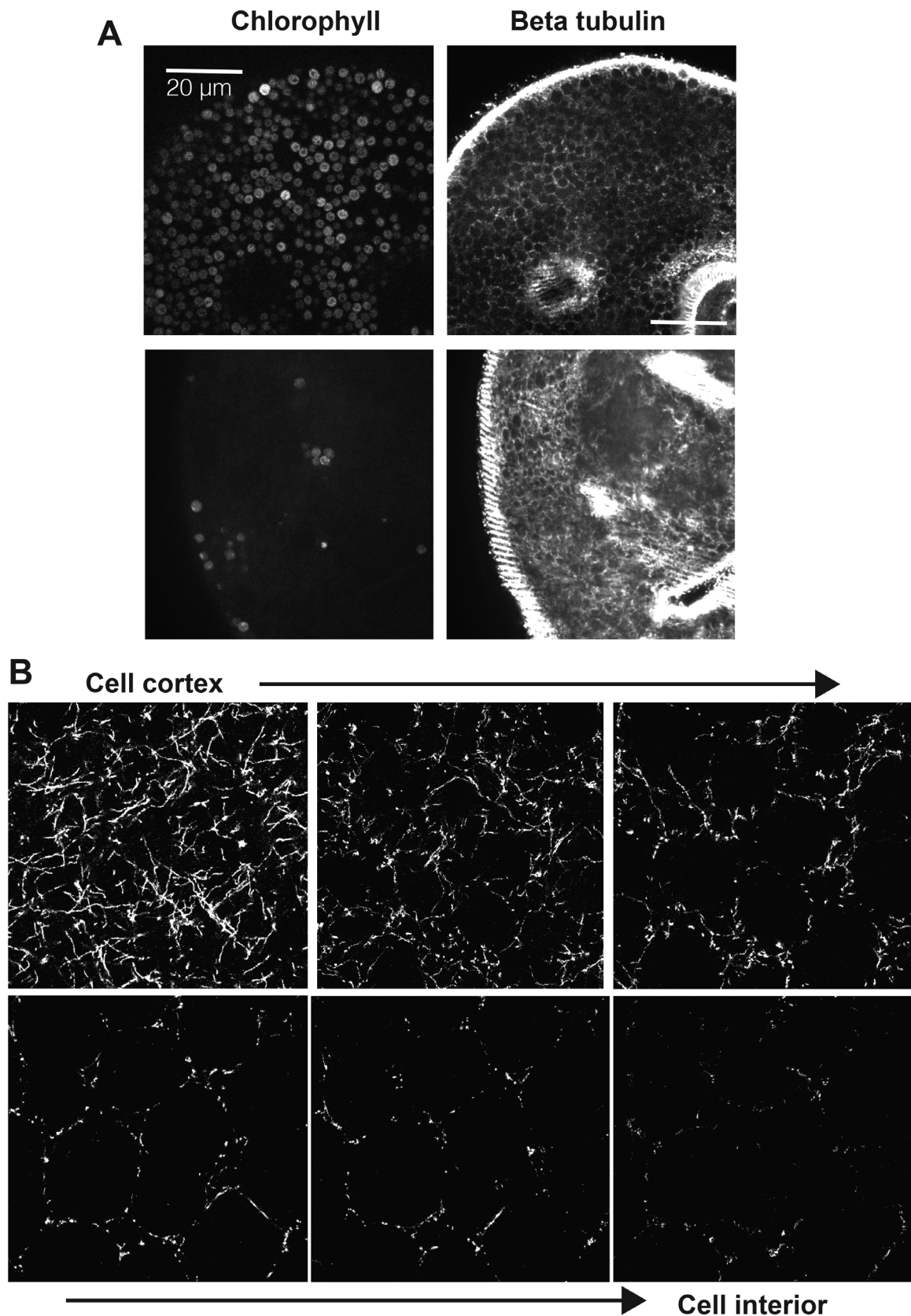


FIGURE 3: Visualization of microtubule baskets on the host cortex surrounding algal symbionts. (A) Chlorophyll autofluorescence of algae (left panels) with immunofluorescence imaging of beta tubulin (right panels) showing *Chlorella* enriched near the cell cortex in a region containing dense microtubules. (B) Expansion microscopy imaging of microtubule baskets. Images show six sequential sections starting from the cell cortex and proceeding inward in 10 μ m steps.

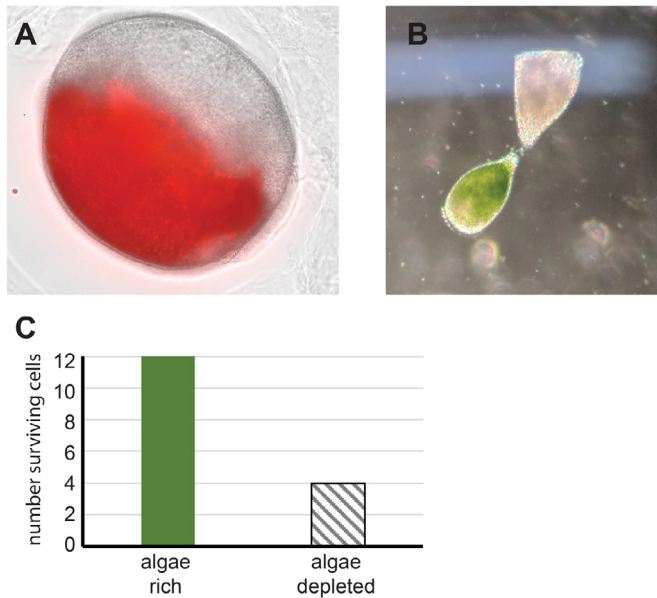


FIGURE 4: Separating host from symbiont. (A) Accumulation of algal endosymbionts on one side of a centrifuged cell as visualized by chlorophyll autofluorescence (red). (B) Example of a cell cleared of algae with a normal cell for comparison. (C) Survival of algae-rich and algae-depleted cells produced by bisection as per panel B. Survival was assessed at 48 h after dissection based on ciliary beating and cytoplasmic integrity.

Cells from which the algae have been removed continue to undergo swimming motion for at least a few hours, but they do not undergo division and eventually die. By 48 h after bisection, the half-cells that retained algae were still fully viable, while the majority of the alga-depleted cells were already dead (Figure 4C). Our experiments do not rule out the possibility that symbiont-free host cells could be kept alive with appropriate nutritional support.

Reduced photosynthetic efficiency in *Chlorella* endosymbiont when inside the *Stentor* host

One of the key questions with respect to endosymbiosis is how, or whether, both the host and the symbiont benefit from their mutual interaction. With this idea in mind, we asked whether the *Chlorella* inside of *S. pyriformis* might show altered photosynthetic efficiency. When a photon is absorbed by chlorophyll, the energy can be used for photochemistry, in which the energy from the photon is 1) used to transfer an electron from the p680 reaction center chlorophyll of photosystem II (PSII) to the primary quinone Q_A , 2) released as heat (nonphotochemical quenching, NPQ), or 3) emitted via fluorescence (Müller et al., 2001). Because fluorescence competes with photosynthesis, it can be used as a measure of photosynthetic efficiency (Baker, 2008).

Pulse-amplitude modulated (PAM) chlorophyll fluorometry is a common and noninvasive method for determining photosynthetic parameters based on chlorophyll fluorescence (Brooks and Niyogi, 2011). By using saturating and photosynthesis-activating actinic light pulses, chlorophyll fluorescence can be induced and quenched. Of critical importance, photosynthetic efficiency can be determined by measuring PSII quantum efficiency, while NPQ can be calculated simultaneously using PAM fluorometry.

The results of our analysis show that the photosystem II efficiency (Φ_{PSII}) of the *Chlorella* cells inside the host cell is ~30% lower than that of algal cells cultured outside the host (Figure

5A). In contrast, our PAM measurements showed a dramatic increase in their capacity for NPQ when the *Chlorella* are inside the host compared with when they are free-living (Figure 5B). One key form of NPQ is energy-dependent quenching, denoted qE, which is a mechanism triggered by high proton concentrations in the thylakoid lumen of the chloroplast. The qE dissipates absorbed light energy from the PSII light-harvesting antenna in the form of heat (Müller et al., 2001). By collapsing the proton gradient across the thylakoid membrane, the proton and potassium ionophore nigericin blocks qE (Brooks et al., 2013). PAM measurements in *S. pyriformis* show that NPQ was blocked by the addition of 15 μ M nigericin (Figure 5B), suggesting that the NPQ largely consists of qE.

Symbioses involving *Chlorella* are typically nitrogen-limited, which is thought to control the proliferation of the symbiont (Reisser, 1976; McAuley et al., 1996; Esteban et al., 2010). Consistent with such a situation in *S. pyriformis*, the high level of NPQ activity that we have detected in *Chlorella* inside *S. pyriformis* only occurs in free-living *Chlorella* when they are nitrogen limited (Figure 5C). The effect is even stronger when low nitrogen is combined with high CO_2 (Figure 5D). Similarly to *S. pyriformis* NPQ, low-nitrogen-stimulated NPQ in free-living *Chlorella* is sensitive to nigericin. Taken together, these experiments suggest that *S. pyriformis* is able to maintain a level of photosynthetic efficiency in its endosymbionts, while activating NPQ mechanisms for photoprotection through nitrogen limitation in its cytoplasm.

Phototaxis in *S. pyriformis* uses directional swimming and requires endosymbionts

We observed that *S. pyriformis* cells tended to accumulate in containers on the side facing the nearest light source, suggesting phototactic behavior. *S. coeruleus*, a *Stentor* species without symbiotic algae, has been shown to exhibit photophobic swimming behavior (negative phototaxis) when exposed to light (Kim et al., 1984). However, when we placed *S. pyriformis* cells into an experimental phototaxis assay (Figure 6A), we found that, similar to other ciliates containing algal endosymbionts such as *P. bursaria* (Matsuoka and Nakaoka, 1988), *S. pyriformis* accumulates near a light source. However, unlike *P. bursaria*, which shows a photoaccumulation behavior in which cells move more slowly in brighter regions so that they gradually accumulate there, *S. pyriformis* swims directly toward light (Supplemental Video S4). During this phototactic motion, we see that the cells clearly orient with their anterior ends toward the light source, and they then swim persistently in that direction.

The motile force for phototaxis is provided by the cilia of the *Stentor* host cells, but which organism, the host or the symbiont, is responsible for sensing the light? Given that nonsymbiotic *Stentor* species can also detect light (Kim et al., 1984), one possibility is that *S. pyriformis* uses a similar phototaxis system, but with different regulatory connections that drive positive rather than negative phototaxis such as is seen in nonphotosynthetic *Stentor* species. Alternatively, it is possible that the algal endosymbionts, which can also detect light, might be serving as the primary photosensors and somehow directing the host cell to swim toward increasing light intensity. To test this idea, *S. pyriformis* host cells were cleared of algae as described in Figure 4, and then tested for phototaxis ability. We found that such alga-free cells no longer undergo phototaxis (Figure 6B), indicating that the presence of algal endosymbionts is required for phototactic motility of the host cell.

To further characterize phototaxis in *S. pyriformis*, we examined its swimming behavior to different wavelengths using illumination

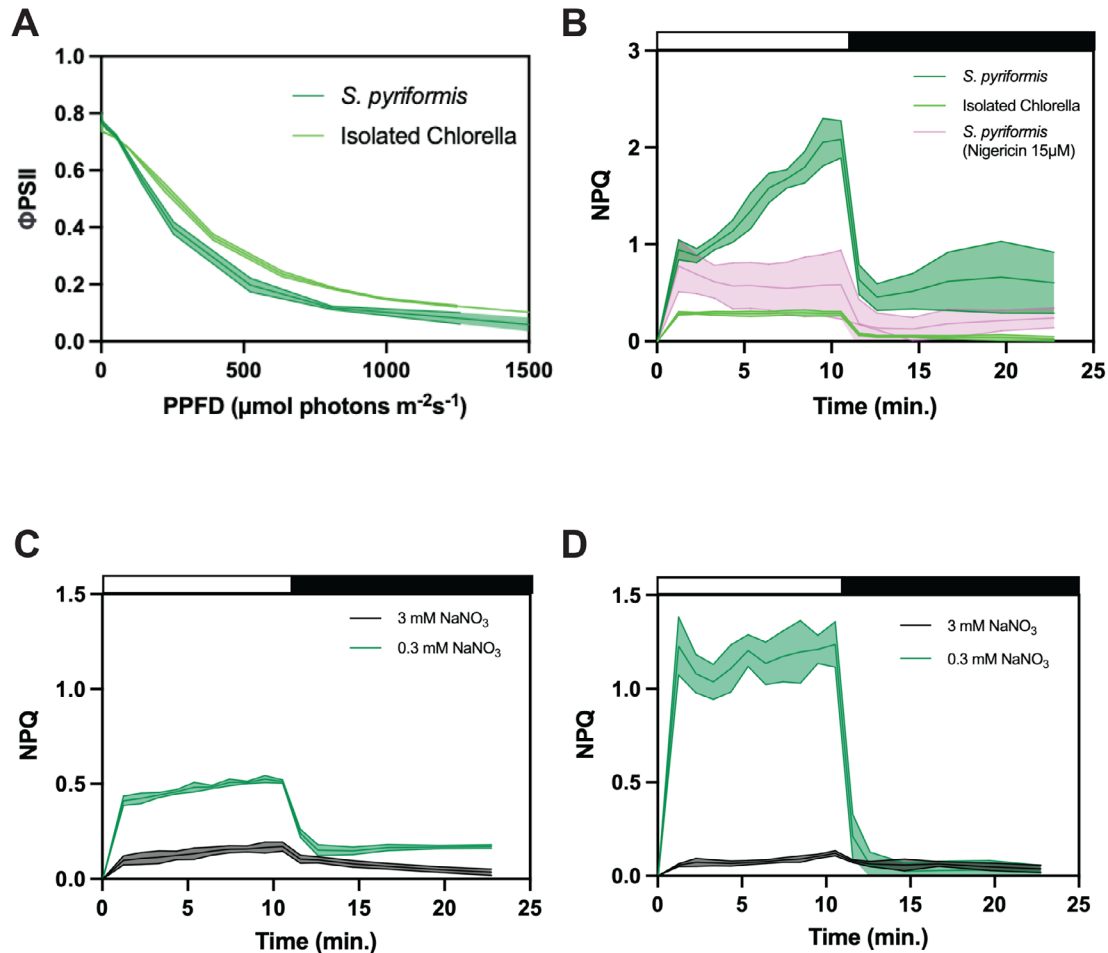


FIGURE 5: Measurement of photosynthetic efficiency of *Chlorella* cells within *S. pyriformis* using PAM fluorometry. (A) Photosystem II operating efficiency (Φ_{PSII}) as a function of photosynthetic photon flux density (PPFD). (B) NPQ as a function of time during and after exposure to actinic light. (C) Increased NPQ in free-living *Chlorella* grown with low nitrogen (0.3 vs. 3 mM NaNO_3) under ambient CO_2 . (D) Further increase in NPQ in free-living *Chlorella* grown with low nitrogen under high CO_2 (3%) conditions. In panels B–D, the white bar above the graph represents exposure to actinic light, and the black bar represents recovery in the dark.

filters (Figure 6, C and D). All land plants and green algae (Chloroplastida) use chlorophylls a and b as light-absorbing molecules in photosynthesis (reviewed in Bhattacharya & Medlin, 1998). Both chlorophylls a and b absorb blue (~425–475 nm) and red (~625–675 nm) light. Interestingly, *S. pyriformis* does not swim at all toward red or orange light (670 or 620 nm) and shows reduced phototaxis toward blue light (485 nm). Instead, *S. pyriformis* prefers swimming toward green light (560 nm). Although a *Stentor*-based green light photoreceptor cannot be ruled out, several green light photoreceptors have been identified in green algae (Nagel et al., 2002).

Finally, we note that phototaxis in *S. pyriformis* obeys a circadian rhythm (Figure 6E) similar to the unicellular alga *Chlamydomonas reinhardtii* (Johnson et al., 1991).

The draft genome of *S. pyriformis*

We assembled the macronuclear genome of *S. pyriformis* using total genomic DNA as described in Materials and Methods. The current assembly is 29.2 Mb of sequence distributed over 203 contigs. We identified telomere sequences using Tandem Repeats

Finder (Benson, 1999) and filtered repeats for all possible permutations of the 8-mer “CCCTAACA.”

We found that 89 contigs have 2 telomeres, 60 have 1 telomere, and 24 have 0 telomeres. In addition, 25 contigs have 3 telomeres, 3 contigs have 4 telomeres, and 2 contigs have 5 telomeres. Additional analyses and curation of the genome are necessary to determine whether these contigs should be split at internal telomere repeats or left intact. In particular, we cannot currently determine whether these represent alternative telomere addition sites that may occur in ciliates (Singh et al., 2023). Excluding contigs shorter than 30 kb, the mean contig length is 185 kb and SD is 51 kb. Including shorter contigs that may be removed or combined after further manual curation otherwise artificially lowers the mean and raises the SD of contig length. As shown in Figure 7A, this length distribution is substantially narrower than that of a draft long-read assembly of *S. coeruleus* (Albright, 2025). We note here that genome comparisons between *S. pyriformis* and *S. coeruleus* are done with the more contiguous draft genome than our published reference genome (Slabodnick et al., 2017). The GC content (35.6% - Figure 7B) is greater than that of *S. coeruleus* (32.2%) and slightly higher than the GC content of the recently sequenced

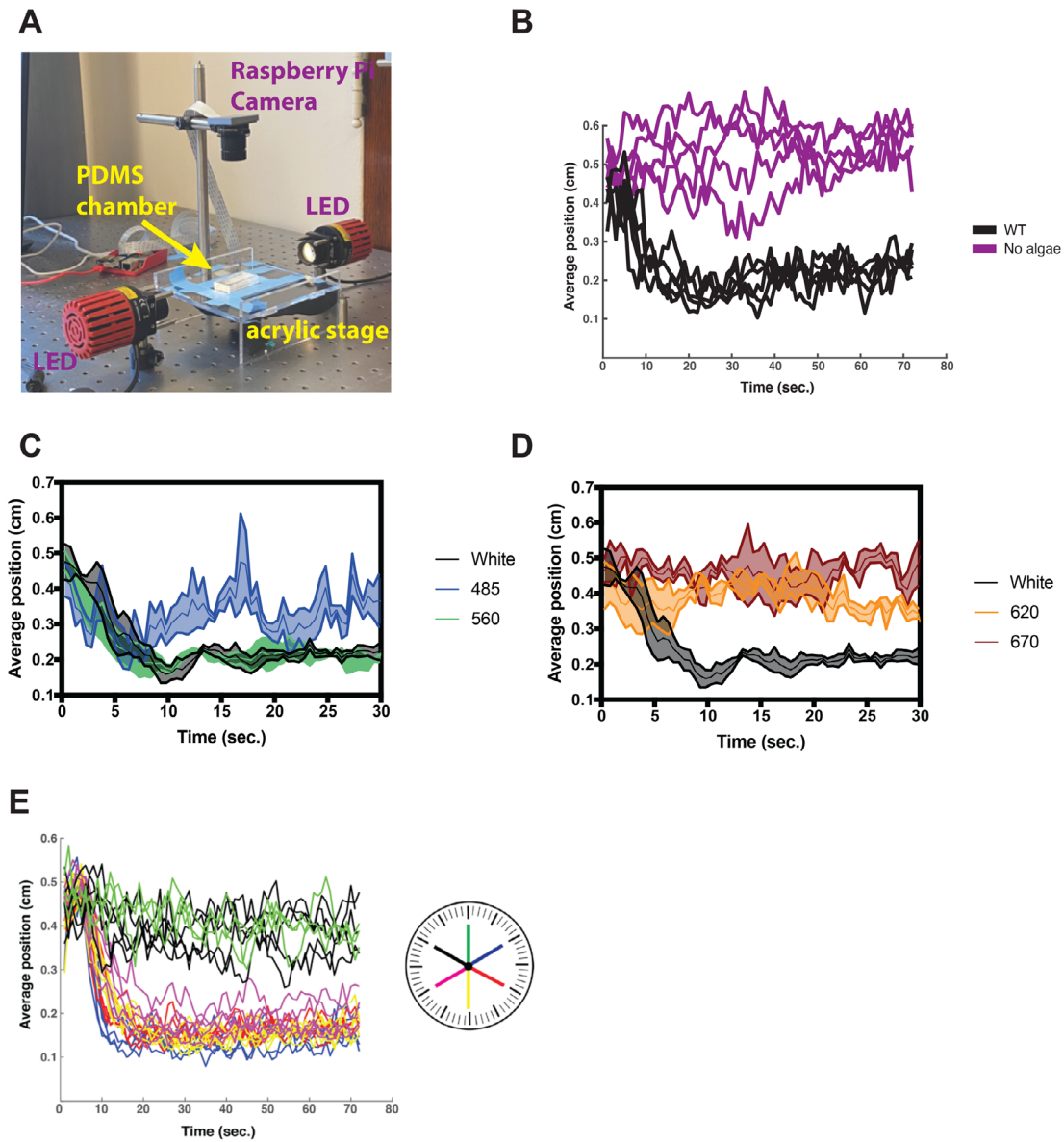


FIGURE 6: Phototaxis in *S. pyriformis*. (A) Apparatus for measuring phototaxis. (B) Phototaxis measured by increased accumulation of cells at one end of the chamber. (black) Untreated cells showing phototaxis. (purple) Cells from which algae were removed, showing loss of phototaxis. (C, D) Phototaxis measured using light of different wavelengths, showing maximal phototaxis for light at 560 nm. (E) Circadian variation in phototaxis. Color code indicates time of day. Cells in these experiments were grown in constant light for a minimum of 2 d prior to the measurement.

Stentor roeselii (34.6% - Zheng et al., 2025). Our phylogenetic comparison of *S. pyriformis* with other *Stentor* genomes (Figure 2F) also suggested that *S. pyriformis* may be more similar to *S. roeselii* than to *S. coeruleus*.

S. coeruleus was notable for its use of extremely small introns, almost all of which were 15 nt in length (Slabodnick et al., 2017). The *S. roeselii* genome also contains almost entirely 15 nt introns (Zheng et al., 2025). To account for short introns in gene prediction, we used Intronarrator (<https://github.com/Swart-lab/Intronarrator>, Singh et al., 2023), which is a wrapper that predicts and removes short introns, runs Augustus with an intronless model, and adds the introns back in the end (see Materials and Methods). We found that while many introns are also short in *S. pyriformis*, there is also a substantial tail of longer introns that was not observed in *S. coeruleus* or *S. roeselii* (Figure 7C).

We note that the contigs we have identified are assumed to represent only the macronuclear genome, given the much higher ploidy compared with the diploid micronucleus. At this point we cannot strictly rule out the possibility that the assembly might include some micronuclear sequence.

***S. pyriformis* macronuclear genes use a standard genetic code**

One unusual feature of most ciliate genomes is their use of non-standard genetic codes, in which some codons specify different amino acids, or mostly notably, where one or more stop codons no longer serve as stop codons but instead are used to encode amino acids, such as UAA or UAG being translated as glutamine or UGA being translated as cysteine (Tourancheau et al., 1995; Lozupone et al., 2001; Swart et al., 2016; Chen et al., 2023). We

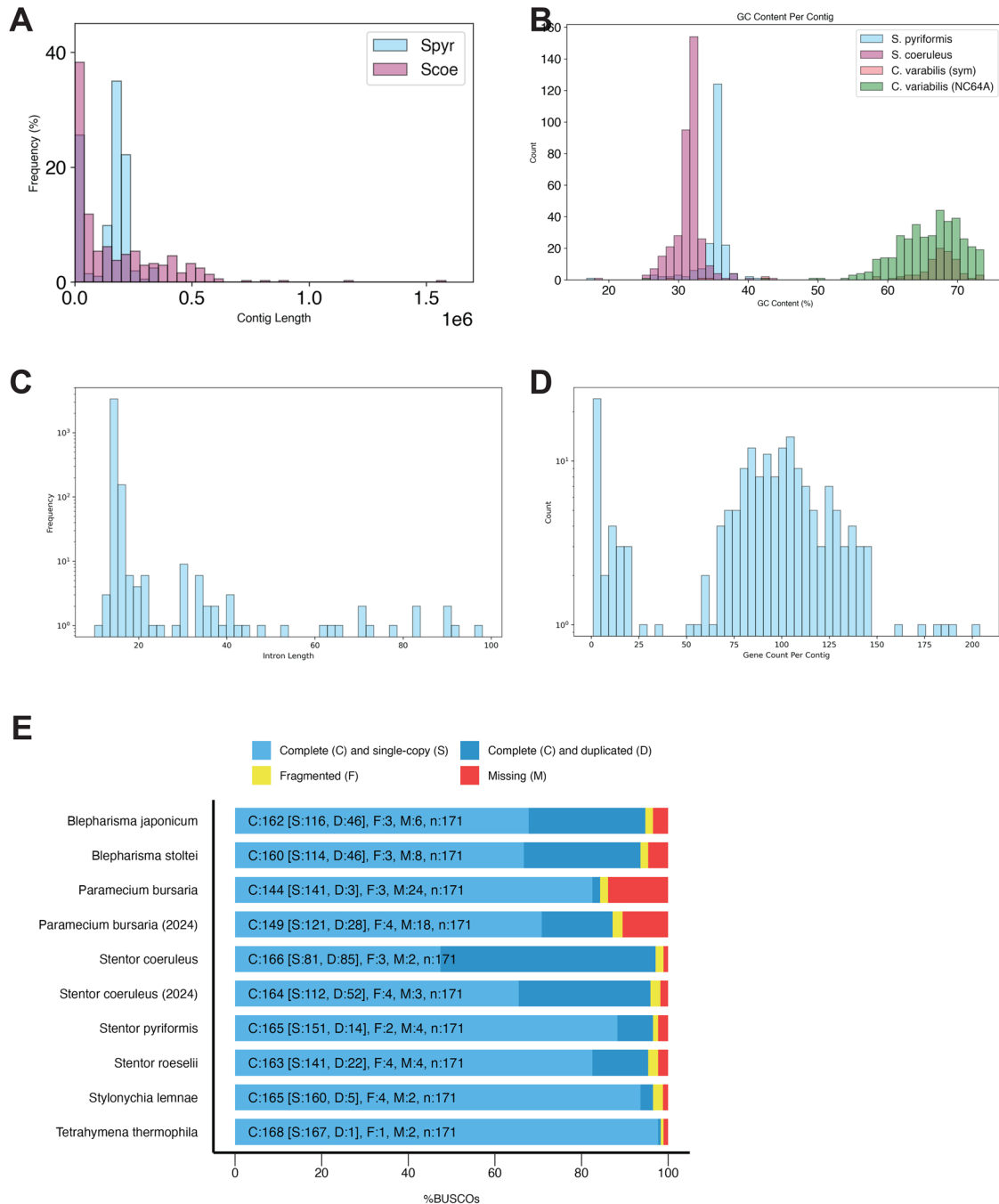


FIGURE 7: Genome of *S. pyriformis*. (A) distribution of chromosome (contig) sizes in assembly of *S. pyriformis* versus *S. coeruleus* genomes. (B) GC content for the genomes of *S. pyriformis* (host and symbiont) compared with *S. coeruleus* and *C. variabilis* NC64A. (C) Distribution of intron lengths. (D) Distribution of predicted gene numbers per contig. (E) BUSCO analysis of *S. pyriformis*.

previously found that *S. coeruleus* uses a standard genetic code in which all three stop codons are used as stop codons (Slabodnick *et al.*, 2017). To determine whether this is also true for *S. pyriformis*, we used Codetta to predict the genetic code (Shulgina and Eddy, 2023). Codetta predictions indicated the use of the standard genetic code; however, Codetta only predicts the genetic code used in coding sequences, and cannot predict stop codon reassignments such as those that are prevalent in ciliates. Thus, we also examined tRNAs predicted by tRNAscan-SE_2.0 (Chan *et al.*, 2021)

which revealed tRNAs for all of the standard amino acids as well as selenocysteine. One tRNA was annotated as a suppressor tRNA, with an anticodon complementary to UGA, but the gene encoding this tRNA mapped to the mitochondrial contig. UGA-targeting tRNA is a shared feature of ciliate mitochondrial genomes (Inagaki *et al.*, 1998) and does not affect the stop codon usage of macronuclear genes. Counts of the final three nucleotides for every predicted coding sequence also showed that all three stop codons were used with different frequencies (UAA 62.0%, UAG 30.5%,

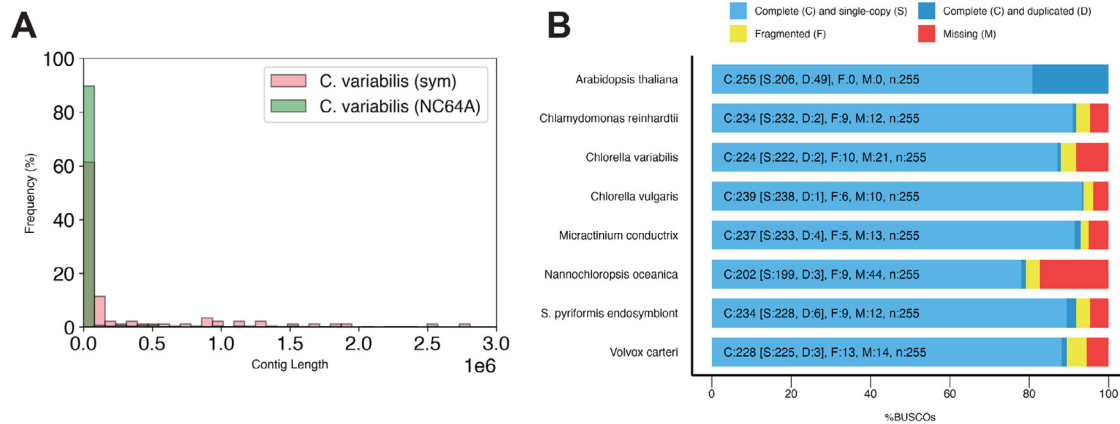


FIGURE 8: Genome of *C. variabilis* isolated from *S. pyriformis*. (A) Distribution of chromosome (contig) sizes in assembly of the *Chlorella* endosymbiont isolated from *S. pyriformis* versus *C. variabilis* NC64A. (B) BUSCO analysis of *C. variabilis* genes and predicted proteins from our isolate of *S. pyriformis*.

UGA 7.5%). Therefore all data indicate that *S. pyriformis*, like *S. coeruleus* but unlike *P. bursaria*, uses a standard genetic code.

Reduced gene complement compared with *S. coeruleus*

The evolution of ciliates has involved a number of whole-genome duplications that help explain the large number of genes found in many ciliates, but it has been noted that in other ciliates with endosymbionts, there are far fewer genes, suggesting less extensive duplication. To ask whether this would also be true in *S. pyriformis*, we predicted gene models from our *S. pyriformis* macronuclear genome assembly as described in *Materials and Methods*, which indicated ~15,000 predicted genes.

Ciliates vary greatly in the organization of macronuclear contigs, with some species having as few as one gene on each contig. Single-gene contigs could potentially have a narrower length distribution than contigs with larger gene numbers. With the predicted gene models in hand, we found that the average predicted number of genes per contig is ~100 (Figure 7D).

One feature of the *P. bursaria* genome is that specific gene families, notably involved in oxygen binding, are depleted relative to other ciliates (He et al., 2019). One explanation for missing genes in a mixotrophic ciliate could be that the presence of the symbiont relieves the host from requiring genes encoding metabolic activities that the symbiont normally provides. To ask whether this might be true in *S. pyriformis*, we carried out a BUSCO (Benchmarking Universal Single-Copy Orthologs) analysis, which is based on a set of highly conserved genes among a wide swath of biodiversity (Manni et al., 2021). BUSCO is primarily intended as a benchmark for genome completeness, but it can also give an indication of genomes that, for reasons such as symbiosis or a parasitic lifestyle, have undergone extensive gene loss. BUSCO analysis of *S. pyriformis* using the *alveolata_odb10* database (Figure 7E), showed a similar number of complete versus missing genes compared with other *Stentor* species that do not contain *Chlorella* endosymbionts, in contrast to *P. bursaria* which clearly has a substantially larger number of missing genes. Given the relatively high quality of the *P. bursaria* genome, this difference may reflect actual gene loss in *P. bursaria* compared with *S. pyriformis*.

The number of predicted *S. pyriformis* genes, approximately 15,000, is substantially smaller than the 34,506 genes annotated in our nonsymbiotic *Stentor* species *S. coeruleus* reference assembly (Slabodnick et al., 2017) and is even smaller than the predicted

gene number in *S. roeselii* (22,896) which has a reduced genome compared with *S. coeruleus* (Zheng et al., 2025). A similar trend is seen with genome size, with the total size of the *S. pyriformis* genome (29.2 Mb) substantially shorter than both *S. coeruleus* (62Mb – Albright, 2025) and *S. roeselii* (45.55 Mb - Zheng et al., 2025). While our annotations of the long-read draft *S. coeruleus* assembly are incomplete, we observe a slight reduction in the percentage of duplicated BUSCOs compared with the assembly (Figure 7E) and believe the number of *S. pyriformis* genes will remain markedly smaller than *S. coeruleus*.

The genome of *C. variabilis* from *S. pyriformis*

We were able to release the *Chlorella* cells into MBBM media (Figure 2C) and culture them on MBBM media agar plates (*Materials and Methods*). We sequenced and assembled the genome of the *Chlorella* symbiont (see *Materials and Methods*) using total DNA isolated from cultured *Chlorella* cells derived from *S. pyriformis* cells. The assembled *Chlorella* genome was 49.6 Mb in size, distributed across 93 contigs and scaffolds with 11,914 predicted genes. For comparison, the best characterized *C. variabilis* species (NC64A) isolated from *P. bursaria* had an assembled genome of 46 Mb, with 12 chromosomes and 9791 predicted genes (Blanc et al., 2010). The GC content of the *Chlorella* from our cells has the same distribution as reported for *C. variabilis* NC64A (Figure 7B). The contig size in our *Chlorella* assembly mostly agrees with that reported for NC64A but has a few larger contigs (Figure 8A). BUSCO analysis indicates a similar completeness of our *Chlorella* genome as that of NC64A (Figure 8B). We note that the *Chlorella* endosymbiont isolated from *S. pyriformis* in Japan (Hoshina et al., 2021) was also closely related to NC64A. One tends to think of the host as being more complex than its endosymbionts, and in this light it is interesting to note that the genome of the *Chlorella* endosymbiont in *S. pyriformis* is substantially larger than the genome of the host itself.

In light of the high NPQ capacity of the *Chlorella* endosymbionts in *S. pyriformis* (Figure 5B), we confirmed that the *Chlorella* genome in our isolates encodes orthologues of chlorophycean violaxanthin de-epoxidase (CVDE) (Li et al., 2016), zeaxanthin epoxidase (ZEP) (Baroli et al., 2003), and stress-related light-harvesting complex (LHCSR) (Peers et al., 2009), all of which are involved in NPQ and photoprotection. LHCSR is required for the rapid induction and relaxation of the qE component of NPQ in the

light-harvesting antenna. CVDE, present in the green algae on the stromal side of the thylakoid membrane, is involved in synthesis of zeaxanthin in high light for NPQ and reactive oxygen scavenging. ZEP is involved in the removal of zeaxanthin in limiting light. All three proteins show high identity with orthologues in related algae (Supplemental Figure S1).

DISCUSSION

Implications of the standard genetic code in *S. pyriformis* for the question of organelle fixation

A notable feature of endosymbiosis in ciliates is the rarity with which endosymbionts become fixed as permanent organelles, with far more cases existing in which the algae retain the ability to live free of the host (Esteban *et al.*, 2010; Johnson, 2011). Such fixation is thought to generally require extensive gene transfer from the endosymbiont to the host, such that the symbiont becomes dependent on the host for the expression of one or more essential genes, and that this dependency eventually results in a symbiont that cannot live apart from the host and is thus considered an organelle. One hypothesis for why such fixation is so rare in ciliates is that most ciliates use a nonstandard genetic code, in which some of the standard stop codons encode amino acids. Our analysis of the *S. coeruleus* genome provided extensive demonstration that *S. coeruleus* uses a standard genetic code, with all three stop codons used as stop codons and no tRNA genes with anticodons that could recognize stop codons (Slabodnick *et al.*, 2017). Here, we show that *S. pyriformis* also uses a standard genetic code. Thus, in this particular case, the explanation for why the symbiont has failed to become fixed as an organelle is unlikely to involve the use of a nonstandard genetic code by the host.

Implications of reduced photosynthesis inside the host

The reduced photosynthetic efficiency of *Chlorella* inside the host (Figure 5) may suggest that the symbiont is gaining some benefit from being inside *Stentor* that outweighs the reduced ability to photosynthesize, or more simply that its photosynthetic efficiency is limited by available nitrogen. We speculate that possible benefits might include protection from predation due to the huge size of a *Stentor* cell, which makes it almost impossible to be ingested by some filter feeders; nutritional supplementation for the alga from the host which, as a filter feeder itself, may have access to food organisms that provide otherwise scarce nutrients that the *Chlorella* can use for growth; and the swimming ability of the *Stentor* host which allows the otherwise immotile *Chlorella* to move from place to place allowing continued access to light. These hypotheses will require direct testing in future experiments. If we grant that the alga is trading reduced photosynthesis for some benefit obtained from the host, could there be any benefit for the host from reduced photosynthesis? Photosynthetic organisms contain mechanisms to protect themselves and their genomes from photo-oxidative damage due to the sometimes intense sunlight to which they are exposed. Compared with nonsymbiotic *Stentor* species, which are best collected in shaded regions of ponds, we routinely find that *S. pyriformis* is abundant near the surface in open regions of water exposed to direct sunlight, either floating near the meniscus or attached to plants near the surface. This spatial distribution is consistent with our observation of positive phototaxis in *S. pyriformis* (Figure 6). However, it poses a potential hazard for the host, whose own genome may become exposed to enough light to produce extensive DNA damage. Our PAM measurements of *Chlorella* photosynthetic activity in *S. pyriformis* indicate that the *Chlorella*

may be collecting and then shunting energy from absorbed light in such a way as to provide photoprotection. Given the spatial location of endosymbionts near the *Stentor* plasma membrane, we speculate that this might serve to help protect the host genome, in addition to protecting the endosymbiont from excess light. Future work could test this idea by removing the algae, exposing the cells to excess light, and measuring the degree of DNA damage. Such an effect has been seen in *P. bursaria*, in which cells cleared of endosymbionts are substantially more sensitive to UV light than when the symbionts are present (Summerer *et al.*, 2009).

P. bursaria has been shown to redistribute its algal endosymbionts when exposed to high light (Summerer *et al.*, 2009). In this case, the algae which are normally located near the cell surface move to a cluster in the posterior region of the cell. Whether this is to provide a denser screen for the benefit of the algae themselves or for host structures like the macronucleus is still uncertain. We have not observed such a rearrangement of endosymbiont position in *S. pyriformis*.

A model for phototaxis in *S. pyriformis* based on oxygen generation by the endosymbionts

A key question concerning phototaxis of *S. pyriformis* is which organism, the host or endosymbiont, is detecting the light. We have found that phototaxis ceases in cells from which the algae have been surgically removed (Figure 6B).

The dependence of phototaxis on the presence of algae is consistent with what has previously been reported in *P. bursaria* and other *Chlorella*-bearing ciliates (Cronkite and van den Brink, 1981; Niess *et al.*, 1981; Finlay *et al.*, 1987; Iwatsuki and Naitoh, 1988). One difference with *S. pyriformis* is that photoaccumulation of *P. bursaria* occurs equally well in red and in blue-green light (Niess *et al.*, 1981), possibly suggesting a difference in the light-sensing mechanism between the two species. Classical algal phototaxis, such as in *Chlamydomonas*, relies on an eyespot with a fixed directional orientation relative to the flagella, such that the cell can change its swimming direction, but in those cases light is sensed by a green light photosensor (Nagel *et al.*, 2002).

The most striking difference between our results in *S. pyriformis* and these prior studies of *P. bursaria* is that, in *P. bursaria*, the photosynthesis-dependent accumulation of cells in regions of bright light does not occur by directed swimming, but by a “kinetic mechanism” (Schnitzer *et al.*, 1990), in which the swimming speed is reduced whenever cells enter a region of higher light intensity, such that cells entering a region of brighter light spend more time there, eventually accumulating in the brightest areas. This effect is mediated by the generation of oxygen by photosynthesis which then regulates ciliary beating (Cronkite and van den Brink, 1981; Iwatsuki and Naitoh, 1988). Similar mechanisms have been reported in other ciliates (Finlay *et al.*, 1987).

In contrast, we have found that *S. pyriformis* swims directly toward the light source, a behavior quite different from the kinetic mechanism of photoaccumulation. One interesting possibility is that local oxygen production by the surface-docked algae could be influencing local ciliary motion in a manner similar to what happens on a whole-cell level in *P. bursaria*. Because of the larger size of *S. pyriformis*, it could be the case that increased oxygen caused by brighter light would cause slower motion of only the cilia near the illuminated part of the cell, causing the cell to steer toward brighter light. This model would require the cilia to respond to oxygen level variations generated by the algae on a time scale comparable to the rotational period of the *Stentor* cell (which is on the order of 1 s). Such a mechanism would require algae to be located near the

cilia, which they are (Figure 1), but would not require the algal cells to be rotationally oriented, which is consistent with our electron micrographs that do not show any preferential orientation of the algal cells with respect either to the cell surface or the long axis of the cell.

Given that the photosensor is inside the algal endosymbionts, but the motile apparatus is clearly the cilia of the host *Stentor* cell, we conclude that phototaxis in this organism thus appears to involve an exchange of information from symbiont to host. It is well known that symbiosis involves back and forth exchange of metabolic products. Phototaxis in *S. pyriformis* supports the idea that the exchange also involves information.

We note that our results only show that the alga and photosynthesis are needed, but this could be a permissive rather than instructive cue. We therefore cannot rule out a model in which the endosymbiont is a permissive cue required to allow phototaxis, but the actual photoreceptor to provide directional information is located in the host cell.

Significance of surface association and microtubule baskets

The location of *Chlorella* cells at the surface of the *S. pyriformis* cell is quite striking, particularly so given the large size of the cells which leaves plenty of room in the interior to accommodate algal cells, as demonstrated by our centrifugation experiments. The retention of the endosymbionts near the surface appears to entail the development of a dense microtubule basket system, not seen in other *Stentor* species, presumably for the primary purpose of symbiont positioning. Could such location on the cell surface confer a fitness benefit to the host or to the symbionts?

The fact that *S. pyriformis* cells rotate while they swim indicates that each algal cell will spend part of the time fully exposed to incoming light and not blocked by any other symbionts, which would not be the case if the symbionts were filling up the interior of the cell. This could potentially help the *Chlorella* cells harvest more light than if they were distributed through the 3D interior of the cell.

Another possible physiological function of surface-docked symbionts could be to provide photoprotection for the host nucleus. By arranging the algae in a shell near the cell surface, with the macronucleus arranged as a small number of round nodes located in the cell interior, photoprotection of the host DNA by NPQ in the algae could be more efficient than if the algae and macronucleus were interspersed with each other, or if the macronucleus was located near the cell surface as it is in other *Stentor* species such as *S. coeruleus*. We note that the arrangement of the alga resembles the arrangement of melanin granules into “umbrellas” that partially surround the nucleus in human skin cells in such a way as to block incoming light (Kobayashi et al., 1998).

Another possible way in which surface docking might be adaptive is that, as discussed above, the association of *Chlorella* cells with the surface near the cilia might help them signal to the cilia to control the direction of cell swimming for phototaxis. Finally, docking near the surface may simply aid with gas exchange between the symbionts and the water outside the host, an effect that seems to explain the docking of mitochondria near the surface of large ciliate cells (Fenchel, 2014).

What cellular adaptations are required for the host cell to be able to locate the endosymbionts near the cell cortex? The extensive meshwork of microtubules at the cell cortex in *S. pyriformis* is not seen in nonsymbiotic *Stentor* species such as *S. coeruleus*, which suggests it may be involved in holding the algae in place. In *P. bursaria*, microtubules play a role in cytoplasmic streaming

(Nishihara et al., 1999), which in turn is required for maximal algal proliferation (Takahashi et al., 2007). The organization of microtubules into a cortical network in *S. pyriformis* suggests a different role, namely in anchoring the algae rather than moving them around. It will be interesting to determine whether other *Stentor* species that contain endosymbionts also display a similar microtubule organization.

S. pyriformis as a model system for unicellular endosymbiosis

Given that much is already known about the cell biology of *Chlorella* endosymbiosis within *P. bursaria*, it is appropriate to ask what we can learn from using *S. pyriformis* as a model system. First, as a ciliate that is highly diverged from *Paramecium* with many unusual features, including a standard genetic code and extremely large cell size, *S. pyriformis* provides a useful comparison. Comparison between cell biological features of endosymbiosis in these two highly diverged ciliates will be especially important if they acquired *Chlorella* endosymbiosis independently, as seems likely to be the case.

The large size of *S. pyriformis* also offers opportunities to learn more about how mixotrophy contributes to aquatic food webs. One of the interesting aspects of mixotrophy is that the host cell can obtain nutrients both from primary production within its endosymbionts and also from predation of other free-living predatory protists, thus tying together two distinct trophic levels. Because of its enormous size, *S. pyriformis* may be able to ingest larger prey compared with smaller mixotrophic ciliates, including other ciliates, colonies of algae or choanoflagellates, or even small animals like tardigrades. Thus this organism takes the crossing of trophic levels to an even greater extreme.

From a technical perspective, the large size and robust wound-healing and regeneration abilities of *Stentor* cells permit micromanipulation experiments that would be difficult in smaller cell types. In particular, the ability to bisect cells after centrifuging all the algae to one side may provide opportunities to study the effect of acute removal of endosymbionts, compared with the more gradual removal that can be achieved in other organisms such as *P. bursaria* using DCMU to inhibit photosynthesis (Reisser, 1976) or cycloheximide to inhibit protein synthesis (Weis, 1984). Because the loss of endosymbionts is gradual in both of those treatments, it may give the host cell time to adapt in a way that it would not be able to do under our acute surgical removal approach, and thus the two types of clearing strategies may provide different types of information about the host–symbiont interaction. Another consequence of the huge size of *S. pyriformis*, which is comparable to the size of leaves in some plants, is that it is so large that PAM fluorometry can be applied to single cells.

Finally, the genus *Stentor* is a classical system for studying cellular morphogenesis, but to date almost all experimental work has been done in just one species, *S. coeruleus*. Further development of *S. pyriformis* will potentially enable an “evo-devo” approach to the study of pattern formation and regeneration in single cells.

MATERIALS AND METHODS

Collection and culturing of *S. pyriformis*

S. pyriformis cells were collected from a pond in Falmouth, MA, by collecting water from near the pond surface, along with submerged plant matter, and then manually picking *S. pyriformis* cells from the water under a dissecting microscope using a pipette. *S. pyriformis* cells were cultured following the same procedure used for culturing

S. coeruleus (Lin et al., 2018), using filtered pond water (Carolina Biological) and feeding the *Stentor* cells with *Chlamydomonas* cells grown in Tris Acetate Phosphate (TAP) medium (Harris, 1989). *S. pyriformis* cells appear to be more sensitive to contamination than *S. coeruleus*, and it proved necessary to remove waste products from the cultures every few days.

Microscopy of *Stentor* cells

For immunofluorescence, antibodies were diluted in antibody dilution buffer (AbDil: 2% BSA in PBS with 0.1% Triton X-100) and stored at 4°C. Cells were picked using a pipette with a cut-off tip and added to fresh MSM (Lin 2018) which was then removed and replaced with fresh MSM. Cells were then starved 12–24 h (overnight) to minimize food vacuoles. The next day, cells were fixed by adding 500 µl ice-cold methanol and incubated for 30 min at –20°C. Excess methanol was then removed by pipetting, followed by incubation in 500 µl of 1:1 PBS:Methanol for 5 min at room temperature, and then in 500 µl PBS for 10 min at room temperature. Cells were then blocked in 500 µl room temperature AbDil for 1 h at room temperature. Primary antibody (anti β -tubulin primary antibody t0198 from Sigma) was added at a dilution of 1:500 in 250 µl of AbDil and incubated for 1 h at room temperature.

After removing excess liquid from the pellet of cells, cell were washed 3×5 min in 500 µl of PBS. Next, 250 µl of secondary antibody diluted 1:500 in AbDil were added along with DAPI diluted 10,000x, and incubated in the dark at room temperature for 1 h. Excess liquid was then removed and cells washed 3×5 min in 500 µl of PBS in the dark, then mounted on a slide and coverslip using Vectashield media.

For electron microscopy, *S. pyriformis* cells were gently pelleted and fixed for transmission electron microscopy with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h at room temperature. After three 5-min buffer washes, cells were postfixed with 1% buffered osmium tetroxide for 1 h, washed in deionized water and dehydrated through increasing concentrations of ethanol (30%, 50%, 75%, 90%, 100%, 100%, 10 min each). Following two changes in propylene oxide (10 min each), the samples were infiltrated in a 1:1 mixture of propylene oxide/Polybed 812 epoxy resin (Polysciences, Inc., Warrington, PA) for 3 h. The pellets were infiltrated overnight in 100% Polybed 812 epoxy resin followed by embedment in fresh epoxy resin and polymerization at 60°C for 24 h. Resin blocks were trimmed to ~2 mm² and sectioned at 70 nm using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Inc., Buffalo Grove, IL) and a Diatome diamond knife (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were mounted on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 10 min and Reynold's lead citrate for 8 min to enhance contrast (Reynolds, 1963). Samples were observed using a JEOL JEM-1230 transmission electron microscope operating at 80 kV (JEOL USA INC., Peabody, MA) and digital images were taken using a Gatan Orius SC1000 CCD camera with Gatan Microscopy Suite version 3.10.1002.0 software (Gatan, Inc., Pleasanton, CA).

Expansion microscopy

We used the U-ExM expansion microscopy protocol (Gambiarotto et al., 2019). *S. pyriformis* cells were adhered to poly-lysine-coated coverslips for 7 min at room temperature, prefixed in 3% formaldehyde in PBS for 10 min at room temperature, washed once in PBS, then fixed in 0.7% formaldehyde with 0.15% or 1% acrylamide in PBS for 4–5 h (with agitation).

A total of 5 µl TEMED and then 5 µl APS were added to 90 µl of MS (19% sodium acrylate [SA], 10% acrylamide [AA - 40%], 0.1% N,N'-methylenebisacrylamide [BIS - 2%] in 1x PBS to yield final concentrations of 0.5% TEMED and 0.5% APS). A total of 35 µl of this mix was then added to each 12 mm coverslip on parafilm.

Polymerization was started at 4°C for 5 min, and then the samples were incubated at 37°C in the dark for 1 h. The gel was then placed in ~2 ml of denaturation buffer (200 mM SDS, 100 mM NaCl, 50 mM Tris in ultrapure water, pH 9 2.88 g SDS, 0.584 g NaCl, 0.303 g Tris in 50 ml MilliQ H₂O, pH 9 with 1 M HCl) in a 6-well plate and incubated for 15 min at room temperature with agitation. Gels were then removed from the coverslips with flat tweezers, moved into a 2 ml Eppendorf centrifuge tube filled with fresh denaturation buffer, and incubated at 95°C for 30 min. After denaturation, gels were placed in beakers filled with ddH₂O for the first expansion. Water was exchanged at least twice every 30 min at room temperature, and then gels were incubated overnight in ddH₂O.

Excess water was then removed by placing the gels in PBS two times for 15 min. Note that in this step, gels shrank back to ~50% of their expanded size. Gels were then incubated with primary antibody diluted in 2% PBS/BSA at room temperature for ~3 h, with gentle shaking.

Gels were then washed in PBS-T three times for 10 min with shaking and subsequently incubated with secondary antibody solution diluted in 2% PBS/BSA for ~3 h at room temperature with gentle shaking. Gels were then washed in PBS-T three times for 10 min with shaking and finally placed in beakers filled with ddH₂O for expansion. Water was exchanged at least twice every 30 min, and then gels were incubated in ddH₂O overnight. We found that the gels expanded between 4.0× and 4.5× according to SA purity.

Gels were then mounted as follows. Each gel was placed in a 10 cm dish and excess water removed with a kimwipe. A small piece of the gel was then cut and transferred smooth side down on a poly-d-lysine (PDL)-coated 35 mm glass bottom dish. The gel was then immobilized with 1% lauryl methacrylate (LMA) and imaged with a 60x n.a. 1.3 oil lens, using a z-step size of 1 µm.

Isolating *Chlorella* from *S. pyriformis*

Approximately 300 *S. pyriformis* cells were collected into a minimal volume of filtered pond water (2 ml total), sonicated until most cells were lysed, and then 500 µl of lysate were layered on top of 1 ml of 60% Percoll and centrifuged at 8k rpm for 1 min. After removing supernatant, the green algal pellet was resuspended in 1 ml of MBBM, centrifuged at 4k rpm for 1 min, and then resuspended in MBBM. *Chlorella* cells were subsequently cultured in MBBM media, both in liquid cultures and on 0.8% agar plates.

Phylogenetic analysis of *S. pyriformis* host and endosymbionts

DNA isolated from *S. pyriformis* cells was used to perform PCR using the Thermo Phire plant direct PCR kit with the following primers:

For *S. pyriformis*: Universal Primers ~500 bp amplicon (Tm: 59°C)

VB017_18S rRNA_F (CG167_18S rRNA_F): CAG CAG CCG CGG TAA TTC C

VB018_18S rRNA_R (CG168_18S rRNA_R): CCC GTG TTG AGT CAA ATT AAG C

For algae in *Stentor*: ~350 bp amplicon (Tm: 65°C)

VB001_18S_rRNA_Pyriformis_F: AAATTAGAGTGTTCAAAGC AGGC

VB002_18S_rRNA_Pyriformis_R: GTCTGGACCTGGTAAGTTT
CCC

For algal *rbcL* sequence (Tm: 61°C)

VB003_RbcL_F: TTACGTTTAGAAGATCTTCGTATTCCAC

VB004_RbcL_R: GCTAATTCAGGACTCCATTGCA

16S rRNA sequencing (Tm: 55°C)

VB015_16s_27f_F: GAGAGTTTGATCCTGGCTCAG

VB016_16s_1492r_R: GGTTACCTGTTCGACTT

DNA sequencing was performed using 200 ng of each PCR product. We generated fasta files containing our 18S or *rbcL* sequences as well as sequences from related species found on NCBI databases. We performed multiple sequence alignment using a ClustalW (Thompson, 1994) web server (<https://www.genome.jp/tools-bin/clustalw>) and trimmed alignments with gblocks (Talavera et al., 2007). We then used the same webserver to run PhyML (Guindon et al., 2010) with 100 bootstraps and used FigTree (<https://github.com/rambaut/figtree/releases/tag/v1.4.4>) for visualization.

PAM measurement of photosynthetic efficiency of *Chlorella* inside the host cell

PAM chlorophyll fluorescence measurements were performed using the Dual-PAM-100 (Heinz Walz) on either *S. pyriformis* or cultured *Chlorella* cells as described previously (Brooks and Niyogi, 2011). *S. pyriformis* and *Chlorella* cell PAM measurements were performed using 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ actinic light and 986 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of saturating light. *Chlorella* PAM measurements were all performed at 5.5×10^5 cells/ml under conditions indicated in the figure legends. *S. pyriformis* PAM measurements were performed using ~300 cells isolated from Morse pond within 24 h, suspended in 2 ml of filtered pond water.

Apparatus for phototaxis measurement

The apparatus in Figure 6A was constructed using LEDs, LED drivers, and LED power supplies from Thorlabs, with bandpass filters to select illumination wavelength and an aspheric condenser lens held with a lens tube (Thorlabs). Images were acquired using a Raspberry Pi HQ camera (16 mm, 5 Mpix) with a CS lens, controlled by a Raspberry Pi 4 desktop kit (4 Gb).

Chlorella genomic DNA extraction

DNA was extracted from *Chlorella* using a procedure modified from methods used for the alga *Nannochloropsis* (Radakovits et al., 2012; Vieler et al., 2012; Gee and Niyogi, 2017). *Chlorella* cells were grown in 50 ml MBBM to midlog phase ($\sim 5 \times 10^6$ to 2×10^7 cells/ml) in a 125 ml sterilized Erlenmeyer flask shaking at ~100 rpm in continuous 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light at constant 28°C. Cells were collected by transferring to 50 ml conical tubes and centrifuging in benchtop centrifuge (1000 rcf) for 5 min, resuspended in 10 ml ddH₂O to rinse away media, and repelleted by centrifugation. Cells were lysed by adding 800 μl of 1x CTAB lysis buffer (2% cetyltrimethylammonium bromide, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA) with 0.8 μl of 100 $\mu\text{g/ml}$ proteinase K (stock solution containing 50 mM Tris (pH 8.0) and 10 mM CaCl₂) to the pellet in 2 ml round-bottom microcentrifuge tubes (MCT), and incubate at 50°C for 30 min to lyse cells and digest proteins. The lysate was then phenol/chloroform extracted using 3.5 ml of 1:1 buffered (pH ~7.5–8.0) phenol and chloroform mixture and centrifuged at 20,000 rcf for 2.5 min. The upper, clear, colorless aqueous phase was transfer to new MCT. RNase A was added to a concentration of 100 $\mu\text{g/ml}$ (8 μl of 10 mg/ml stock) and incubated at 37°C for 60

min. The phenol/chloroform extraction was then repeated, transferring to another clean MCT.

Two final extractions were then performed with 100% chloroform to remove trace phenol using same volumes and spins. After last extraction, 550 μl was transferred to fresh MCT to avoid collecting the interface. DNA was then ethanol precipitated by adding 2.5 volumes (e.g., 1.375 ml) of ice-cold 100% EtOH and 0.1 volume of 3M sodium acetate (e.g., 55 μl). After precipitation for 60 min at –20°C, tubes were centrifuged at 4°C for 15 min at 20,000 $\times g$. The pellet was washed with 1 ml ice-cold 70% EtOH and centrifuged again for 5 min max speed at 4°C to make sure pellet was seated securely again.

The pellet was then dried in a stream of filtered air, and resuspended in 30 μl of water. DNA quality was checked by Nanodrop spectrophotometry followed by a fluorometric Qubit assay.

Sequencing of the *S. pyriformis* and *C. variabilis* genomes

Total genomic DNA samples from *S. pyriformis* were prepared using the DNeasy Blood and Tissue kit (Qiagen), following the suspension cell protocol, as described previously (Slabodnick et al., 2017). Genomic DNA was sequenced using an Oxford Nanopore MinION, R9.4 flow cell, and ligation sequencing prep (LSK109), which produced 0.5 million reads with an N50 size of 6 kbp and a total of 1.7 Gbp of sequence. Nanopore sequencing reads were assembled using Flye (Kolmogorov et al., 2019) followed by polishing with Medaka (nanoporetech.com).

No algal reads were obtained during the sequencing and assembly process using DNA obtained from *S. pyriformis* cells, which is to be expected given the difficulty of breaking algal cell walls. For this reason, DNA was isolated from *Chlorella* cells obtained from *S. pyriformis* and cultured on MBBM plates. Sequencing and assembly of the *Chlorella* genome was performed the same as for *S. pyriformis*.

Nanopore sequencing genomic DNA from *S. pyriformis* and its endosymbiont, *C. variabilis* is available at Data Dryad (Albright et al., 2025).

The genome assembly for both *S. pyriformis* and its *Chlorella* endosymbiont presented in this paper are available online at StentorDB (Stentor.clate.org).

RNA-seq and gene prediction for *S. pyriformis*

Full code and descriptions for RNA sequencing (RNA-seq) alignment and gene prediction, including data on intron lengths and contig sizes, are available at https://github.com/ArAlbright/2022_pyriformis. We altered the source code of HISAT2 (Kim et al., 2019) to accommodate for short introns as found in other heterotrichs (Singh et al., 2023), and the closely related *S. coeruleus* (Slabodnick et al., 2017). The variable minIntronLen in hisat2.cpp was reduced to 9. HISAT2 with this change was run with the following parameters: –min-intron-len 9 –max-intron-len 101 –very-sensitive. We used samtools (Li & Durbin, 2009) to sort and index the alignments.

Genes were predicted using Intronator (<https://github.com/Swart-lab/Intronator>), which predicts and removes introns before running Augustus with an intronless model and adds back the introns at the end. We ran Intronator by altering the path variables in intronator.sh as well as the following parameters: MAX_INTRON_LEN = 101 and GENETIC_CODE = 1. To standardize the gene names, we modified a GFF Parser (Hannon and Eisen, 2024) and added custom scripts to change the names of contigs to StePyr_contig_# and genes to StePyr_#.

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